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Dynamic expression of MMP28 during cranial morphogenesis

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Matrix metalloproteinases (MMPs) are a large family of proteases comprising 24 members in vertebrates. They are well known for their extracellular matrix remodelling activity. MMP28 is the latest member of the family to be discovered. It is a secreted MMP involved in wound healing, immune system maturation, cell survival and migration. MMP28 is also expressed during embryogenesis in human and mouse. Here, we describe the detailed expression profile of MMP28 in *Xenopus laevis* embryos. We show that MMP28 is expressed maternally and accumulates at neurula and tail bud stages specifically in the cranial placode territories adjacent to migrating neural crest cells. As a secreted MMP, MMP28 may be required in neural crest–placode interactions.

This article is part of a discussion meeting issue 'Contemporary morphogenesis'.

1. Background

Matrix metalloproteinases (MMPs) form a large family of proteases containing 23 members in human and 24 in *Xenopus laevis* [1]. MMPs are translated as zymogens, or pro-enzymes, where the N-terminal part of the protein, called the pro-domain, blocks the catalytic domain. This pro-domain can either change conformation (a process known as allosteric activation) or be removed by proteolytic cleavage to ensure the activity of the protein [2]. MMPs are Zn^{2+}-dependent proteases mostly known for their extracellular matrix remodelling activity but they are also capable of cleaving a wide range of substrates including growth factors and their cognate receptors, adhesion molecules and chemokines [3]. For decades, MMPs have been studied in the context of cardiovascular diseases, rheumatoid arthritis and neurological disorders. MMPs are also of prime interest in cancer, since MMPs are present in virtually all human cancers, and most members of the MMP family have been found to be dysregulated in human cancers [4].

MMPs are essential for key cellular processes from extracellular matrix regulation to cell migration and invasion to cell proliferation and apoptosis. Therefore, they are important in adults for wound healing, angiogenesis, tissues homeostasis and immunity. In addition, MMPs are expressed during embryogenesis and are involved throughout development [5]. Indeed, about half of all MMP family members are expressed during *Xenopus laevis* embryogenesis (table 1).

MMP28 was first identified in human and is the latest member of the family to be discovered [15,16]. MMP28 is a soluble MMP with endopeptidase activity and the ability to degrade casein, N-CAM and Nogo-A proteins [13]. MMP28 is involved in nerve repair and wound healing, immune system maturation, cell migration and invasion [17,18]. MMP28 was detected by PCR in human fetal tissues including skeletal muscle, lung, kidney and brain, and by *in situ* hybridization in mouse heart, central nervous system and peripheral nervous system from stage E.10 onward [13,16,19]. In early *Xenopus* embryogenesis, MMP28 was first identified in a comparative microarray screen performed on dissected neural border explants to identify neural crest (NC)-specific transcripts [12]. NC cells arise from the neural plate border alongside the cranial placodes. They give rise to a wide variety of derivatives, including smooth muscles, melanocytes, craniofacial bones and

Table 1. Developmental expression and putative substrates of matrix metalloproteinases identified in *Xenopus laevis*. St., embryonic stage.

name	type	localization	known substrates	reference(s)
MMP2	soluble	St. 20–40: migrating NC, head, pharyngeal arches, mesenchyme, periocular region	collagen I, II, III, IV, V, VII, X, XI and XIV, gelatin, elastin, fibronectin, laminin, aggrecan, versican, osteonectin, proteoglycans, nidogen, MMP9 and 13, FGFR-1, IGFBP-3 and 5, IL-1β, Pro TNFα, TGFβ	Tomlinson *et al.* [6], Xenbase
MMP3	soluble	St. 29–30: epidermis, fin, cloaca	collagen III, IV, V and IX, gelatin, aggrecan, perlecan, decorin, laminin, versican, proteoglycan, tenascin, fibronectin, osteonectin, elastin, casein, ovostatin, entactin, plasminogen, MBP, IL-1β, MMP2/TIMP-2, MMP7, MMP8, MMP9, MMP13	Xenbase
MMP7	soluble	St. 17–34: myeloid cells, epidermis, macrophage	collagen IV and X, gelatin, aggrecan, decorin, fibronectin, laminin, elastin, casein, transferrin, plasminogen, MBP, β-4 integrin, MMP1, MMP2, MMP9, MMP9/TIMP-1	Harrison *et al.* [7], Tomlinson *et al.* [8], Xenbase
MMP9	soluble	St. 24–46: epidermis, macrophage	collagen IV, V, VII, X and XIV, gelatin, elastin, aggrecan, versican, proteoglycan, osteonectin, fibronectin, laminin, CXCL5, IL-1β, IL2-R, pro-TNFα; TGFβ, SDF-1	Tomlinson *et al.* [8], Xenbase
MMP11	soluble	St. 32–45: pharyngeal arches, rectal diverticulum, head ectoderm, eye, endoderm, notochord, cloaca, pharynx, pharyngeal epithelium, fibroblast, fin, intestine connective tissue	casein, laminin, fibronectin, gelatin, collagen IV, transferrin	Damjanovski *et al.* [9], Xenbase
MMP13	soluble	St. 33–41: head, pharyngeal arches, endoderm, dorsal lateral plate mesoderm, pharyngeal epithelium	collagen I, II, III, IV, IX and XIV, gelatin, tenascin, fibronectin, aggrecan, osteonectin, plasminogen, perlecan, MMP9	Damjanovski *et al.* [9], Xenbase
MMP14	transmembrane	St. 10–34: migrating neural crest, cephalic neural crest, trunk neural crest, neural tube, pharyngeal arches, proctodeum, R3 and R5, notochord, dorsal marginal zone	proMMP-2, proMMP-13, ADAM9, gelatin, collagen I, II and III, fibronectin, vitronectin, laminins-1, -2/4 and -5, fibrin/fibrinogen, α1PI, perlecan, CD44, ICAM-1, tTG, LRP1, syndecan 1, αv-integrin, C3b, EMMPRIN, ApoE, MICA, β-glycan, IL-8, SLP1, CTGF, DR6, DJ-1, galectin-1, Hsp90α, pentraxin 3, progranulin, Cyr61, peptidyl-prolyl *cis-trans* isomerase A, dickkopf-1, KiSS-1, DII, SDF-1	Harrison *et al.* [7], Xenbase

(*Continued*.)

Table 1. (*Continued.*)

name	type	localization	known substrates	reference(s)
MMP15	transmembrane	St. 0–34: ventral ectoderm	proMMP-2, proMMP-13, fibronectin, aggrecan, vitronectin laminin-1, nidogen, perlecan, collagen I, NC1 (coll-IV), fibrin	Harrison *et al*. [7], Xenbase
MMP16	transmembrane	St. 25–36: eyes, neural tube, somites, otic vesicles	activates proMMP2 with TIMP2, proMMP-2, gelatin, collagen III, laminin-1, fibronectin, vitronectin, fibrin, α1PI, NgR1, KiSS-1, aggrecan, perlecan	Hammoud *et al*. [10]
MMP18	soluble	St. 24 and 45: cornea, eye, foregut, head endoderm, intestine, limb, notochord, pharyngeal arches, tail, epidermis, macrophage	collagen I, II and III, gelatin	Tomlinson *et al*. [8], Xenbase
MMP20	soluble	St. 66: upper jaw	amelogenin, aggrecan, COMP	Shintani *et al*. [11], Xenbase
MMP24	soluble	St. 35–40: retina, brain, cement gland, spinal cord	proMMP-2, gelatin, fibronectin, laminin-1, CSPG, DSPG, KiSS-1, N-cadherin	Xenbase
MMP28	soluble	St. 12.5–50: neural crest, cranial placode, neural plate border, peripheral nervous system	N-CAM, Nogo-A, casein	Plouhinec *et al*. [12], Werner *et al*. [13], Xenbase, this study
RECK	GPI-anchored	St. 18–38: anterior face, eye, pharyngeal arches, anterior notochord, neural tube		Willson *et al*. [14]
TIMP-1	soluble	St. 15–28: myeloid cells, ventral blood island		Xenbase
TIMP-3	soluble	adult		Xenbase

cartilage, and together with placode cells, all the peripheral nervous system of the head [20]. So far, MMP2, 11, 13, 14 and 16 have been described as being expressed by the NC or by tissues surrounding the NC (see table 1). However, a detailed expression profile of MMP28 during *Xenopus* development is still lacking.

In this study, we used qPCR analyses, and single and double *in situ* hybridization to assess the expression of MMP28 during *Xenopus laevis* embryogenesis. We found that MMP28 has a well-defined expression domain in the pre-placodal region at early neurula stage. During NC cell migration, MMP28 is then detected in some placodes and NC subpopulations. Given that NC and placodes interact to form the cephalic peripheral nervous system [20] and that MMP28 is secreted, we propose that MMP28 may play a role in mediating these interactions to direct cranial morphogenesis.

2. Material and methods

(a) *Xenopus* manipulation and *in vitro* fertilization

All experiments were performed in accordance with the guidelines of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and were approved by the Institutional Animal Care and Use Committee of New York University (animal protocol no. IA16-00052). Female *Xenopus laevis* were injected with 500–1000 units of chorionic gonadotrophin and kept overnight at 18°C. For fertilization, a suspension of minced testis was added to the oocytes collected in a Petri dish in 0.1× normal amphibian medium (NAM): NaCl (110 mM), KCl (2 mM), $Ca(CO_3)_2$ (1 mM), $MgSO_4$ (1 mM), EDTA (0.1 mM), $NaHCO_3$ (1 mM), sodium phosphate (2 mM). Embryos were staged according to Nieuwkoop & Faber [21].

(b) qPCR

Embryos were collected by quick-freeze into liquid nitrogen. Total RNAs from batches of three to five embryos were extracted with the RNeasy Micro Kit (Qiagen, Valencia, CA). Relative quantitative PCR was performed on a QuantStudio 3 Real-Time PCR System (Applied Biosystems, Foster City, CA) using the Power SYBR Green RNA-to-C_T 1-Step Kit according to the manufacturer's instructions and the following primers: Twist1qPCR_fdw: 5′-CGACTTTCTCTGCCAGGTCT-3′; Twist1q-PCR_rev: 5′-TCCACACGGAGAAGGCATAG-3′; MMP28-E7_fdw: 5′-TGCAGTGGTATCGGGTTTAG-3′; MMP28-E8_rev: 5′-AAAGTGCAGTGTCAGGACGA-3′; Sox10_fdw: 5′-CTGTGAA-CACAGCATGCAAA-3′; Sox10_rev: 5′-TGGCCAACTGACCATG TAAA-3′; Six1_fdw: 5′-CTGGAGAGCCACCAGTTCTC-3′; Six1_rev: 5′-AGTGGTCTCCCCCTCAGTTT-3′; ODC_fdw: 5′-ACATG

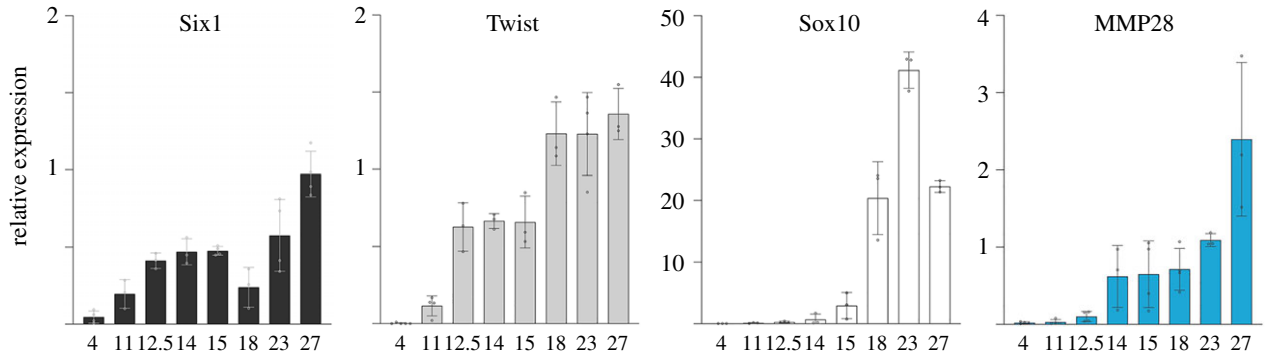

Figure 1. Expression of MMP28 assessed by qPCR. Relative expression of Six1 (black), Twist (light grey), Sox10 (white), MMP28 (blue) during *Xenopus* embryogenesis from stage 4 to stage 27. Results were generated using the ΔC_T method with the house-keeping gene ODC as reference and expressed as mean of at least three independent biological samples; error bars correspond to standard deviations. For representation purposes, relative expression value results were multiplied by 1000. (Online version in colour.)

GCATTCTCCCTGAAG-3′; ODC_rev: 5′-TGGTCCCAAGGCTAAAGTTG-3′. All primer pairs were validated using the standard curve method. Data from embryos collected from three different females were normalized to ODC using the ΔC_T method [22] and expressed as mean with standard deviation error bars.

(c) *In situ* hybridization and histology

Embryos were fixed for 1 h at room temperature in MEMFA (0.1 M MOPS pH 7, 42 mM EGTA pH 7.0, 1 mM $MgSO_4$, 3.7% (wt: vol) formaldehyde) and stored in 100% methanol. Embryos were then rehydrated in methanol solutions of decreasing concentrations, and processed for single or double *in situ* hybridization as previously described [23]. The following probes were used: Xl-Snai2 [24], Xl-MMP28 (this study), Xl-Sox10 [25], Xl-Six1 [26], Xl-Foxi4.1 [27], Xl-Sox2 [28] and Xl-Dmrta1 [29]. Each staining was performed at least three times on batches of embryos collected from different females. For histology, stained embryos were embedded in Paraplast Plus, sectioned (12 µm) on an Olympus rotary microtome, counter-stained with eosin and mounted in Permount.

3. Results

Xenopus has two homologous copies [30] of MMP28 gene on chromosomes 2L (MMP28.L) and 2S (MMP28.S), which are composed of eight exons each and encode a 1977 and 1916 bp mRNA, respectively. Both mRNA sequences share 84.71% identity, and 92.9% identity for the open reading frame. MMP28.L and MMP28.S code for proteins of 496 and 497 amino acids, respectively, with a predicted molecular weight of 57 kDa. The two protein sequences share 91.15% identity and 97.58% similarity. As the sequences have high identity, we designed primers and probes for *in situ* hybridization that detect both MMP28.L and MMP28.S.

We assessed the temporal expression of MMP28 by relative qPCR at different stages of *Xenopus* development from stage 4 (8-cell stage) to tail bud stage, and compared its expression profile with that of Twist and Sox10, two markers of cephalic NC [25,31], and Six1, a marker for cranial placodes [32] (figure 1). MMP28 is maternally expressed (stage 4) although at relatively low levels, similar to Six1, while Twist and Sox10 do not appear to have a maternal contribution. After the mid-blastula transition, the expression of embryonic genes is initiated. MMP28 expression levels remain relatively low at stage 11 and stage 12.5 similar to Sox10, while both Twist and Six1 expression increase significantly during that period until stage 15 when they both transiently reach a plateau of expression. MMP28 shows a marked increased expression at stage 14, which is maintained until stage 18. Later in development, MMP28 expression progressively increases up to stage 27, the last stage examined in this study. Sox10 expression increases strongly between stages 15 and 23 before declining at stage 27. Twist reaches its highest expression level at stage 18 without any further increase later in development. Six1 expression decreases significantly at stage 18 and at later stages progressively increases in a similar manner to MMP28. These data indicate that MMP28 expression is strongly upregulated at the neurula stage (stage 14) and the escalation of Twist and Six1 expressions precedes that of MMP28 and Sox10.

We next performed *in situ* hybridization to characterize the spatial expression of MMP28 during *Xenopus* development. MMP28 is not detected at gastrula stage (figure 2*b*,*c*). MMP28 transcripts first accumulate at detectable levels at the end of gastrulation (stage 12.5), in the form of a thin horseshoe-shaped domain at the anterior part of the embryo, which represents the neural plate border (figure 2*e*), the region that gives rise to both cranial placodes and NC cells. A control sense probe confirmed the specificity of this expression (figure 2*d*). At stage 14/15, MMP28 expression is more defined into two bilateral domains on either side of the neural plate (figure 2*f–h*). This staining strongly suggests a placodal expression since the neural fold is devoid of staining. At this stage, MMP28 is also detected in a more discrete domain along the border of the neural plate (figure 2*h*,*i*′, arrowheads). To substantiate these observations, stage 15 embryos were sectioned to visualize MMP28 expression in more detail (figure 2*h*,*h*′,*h*″,*h*‴). MMP28 expression is localized in the deep ectoderm layers, consistent with cranial placode expression (figure 2*h*‴,*i*′, arrows). By contrast, the thin line of expression along the neural plate is localized in the most superficial ectoderm layer (figure 2*h*‴,*i*′, arrowheads), which presumably corresponds to the medial NC [33,34]. At stage 17, the expression is broader and outlines the prospective eyes (figure 2*h*‴,*j*, arrowhead). At stage 23, during NC cell migration, MMP28 expression seems to be restricted to discrete domains around the eyes and in territories posterior to that region, likely to correspond to the epibranchial placodes (figure 2*l*, *l*′). At stage 25, strong MMP28 expression is detected in the first stream of NC as

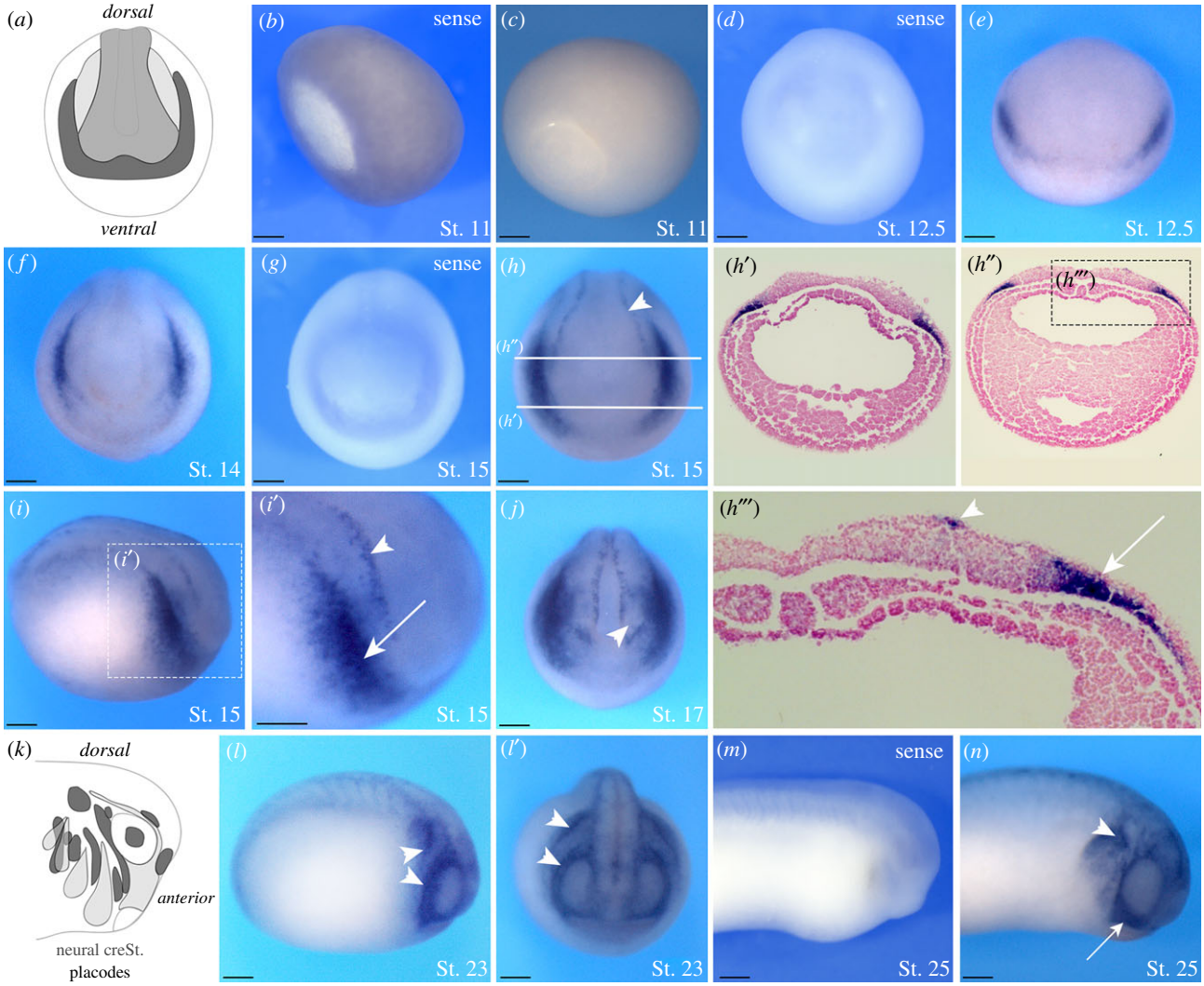

Figure 2. Developmental expression of MMP28 by *in situ* hybridization. *In situ* hybridization for MMP28 in lateral view (*b*, *c*, *i*, *i*′, *l*, *m*, *n*), anterior view (*d*,*e*, *j*, *l*′), dorsal view (*f*–*h*) and transversally sectioned embryos (*h*′ and *h*″). (*a*,*k*) Schematic of placodes (dark grey), neural crest (light grey) and neural plate (mid-grey). (*b*,*c*) At St. 11 MMP28 is not detected. (*d*,*e*) MMP28 is first detected at the anterior neural plate border at St. 12.5. (*f*–*j*) Neurula stage embryos show expression in the lateral placodes (arrows) and medial NC (arrowheads). (*h*′–*h*‴) Transverse sections as indicated in (*h*), placodes (arrow), medial NC (arrowhead). (*l*,*l*′) Expression in the epibranchial placodes (arrowheads). (*m*,*n*) At tail bud stage MMP28 expression is strongly detected in the first NC stream (arrow) and epibranchial placodes (arrowhead). The embryonic stage (St.) is indicated in the lower right corner of each panel. When the MMP28 sense probe is used (sense) it is indicated in the upper right corner of the panel. Scale bars, 0.25 mm. Number of embryos per experiment greater than 20.

well as what appear to be the third and fourth NC streams (figure 2*n*, arrow). The specificity of MMP28 expression was confirmed using a control sense probe (figure 2*m*).

In order to identify more precisely which tissues express MMP28, we performed *in situ* hybridization at stages 15 and 25 for MMP28 and genes expressed in similar regions of the ectoderm, including Sox2 (neural plate and cranial placodes), Snai2/Slug (NC), Dmrt1a (olfactory placodes), Foxi4.1 (epibranchial placodes) and Sox10 (migrating NC). The probes were used alone or in combination as indicated (figure 3). Spatially, there is no overlap between MMP28 and Snai2, as the Snai2 expression domain (figure 3*a*,*a*′) is medial to that of MMP28 (figure 3*g*′,*b*,*g*). Sox2 is expressed in two regions, the neural plate (figure 3*c*, arrow) and lateral placodes (figure 3*c*,*c*′, arrowhead). MMP28-positive territory overlaps exclusively with the Sox2 placodal expression domain (figure 3*d*,*d*′). Since cranial placodes cover broad area giving rise to different derivatives, we also compared MMP28 with Dmrta1, a gene restricted to the most anterior placodal region, the prospective olfactory placodes (figure 3*e*,*e*′). We found that MMP28 and Dmrta1 are expressed in adjacent but non-overlapping domains (figure 3*f*,*f*′). At stage 25, Foxi4.1 is strongly expressed in the epibranchial placodes and sensory layer of the ectoderm (figure 3*j*, arrowheads) but not in the NC streams (figure 3*j*, arrows). By contrast, Sox10 is expressed strongly in all NC streams (figure 3*k*, arrows) and excluded from the branchial ectoderm. This histological analysis indicates that MMP28 is not expressed in the posterior NC streams (figure 3*l*, arrows) but shows clear expression in the epibranchial placodes and sensory layer of the ectoderm (figure 3*l*, arrowhead).

Our results indicate that, at the neurula stage, MMP28 is not expressed in the NC but is confined to the cranial placodes territory, overlapping with Sox2 (figure 3*h*) and that its expression is maintained in the epibranchial placodes and sensory layer of the ectoderm at the tail bud stage (figure 3*l*).

4. Discussion

Here, we report the developmental expression of MMP28, a member of the MMP family, in *Xenopus*. Our results showed that MMP28 is expressed maternally even though faintly and

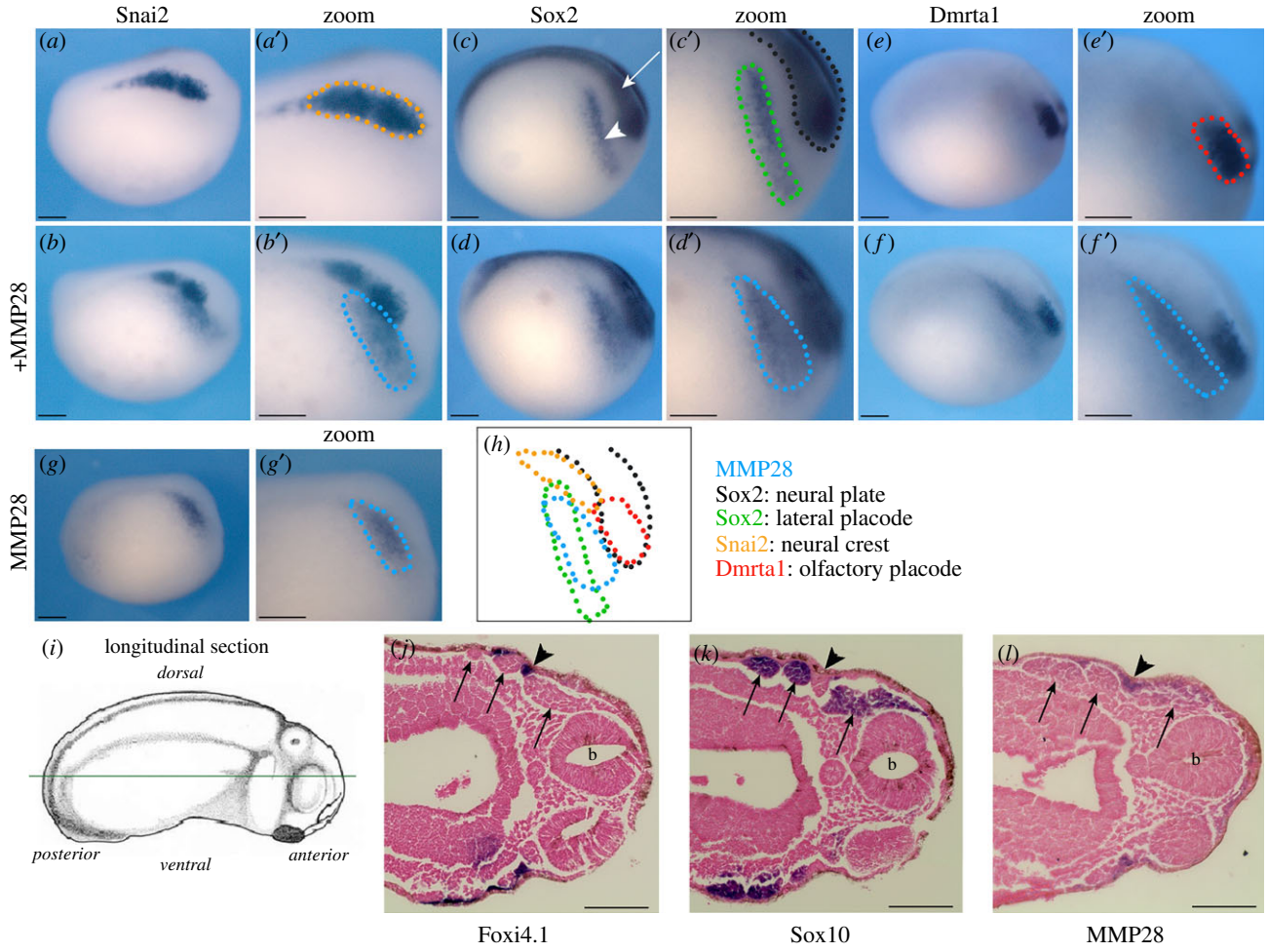

Figure 3. Comparative analysis of MMP28, Snai2, Sox2, Dmrta1, Foxi4.1 and Sox10 expression by single and double *in situ* hybridization. (*a–h*) *Xenopus* embryos at stage 15 (lateral view, anterior to right, dorsal to top) after single *in situ* hybridization for Snai2 (*a*,*a*′), Sox2 (*c*,*c*′), Dmrta1 (*e*,*e*′) and MMP28 (*g*,*g*′), or double *in situ* hybridization for MMP28/Snai2 (*b*,*b*′), MMP28/Sox2 (*d*,*d*′) and MMP28/Dmrta1 (*f*,*f*′). Summary of the expression domains of these genes (*h*). Dotted lines represent expression territories of MMP28 (blue), Snai2 (orange), Sox2 (black, neural plate; green, placodes) and Dmrta1 (red). MMP28 (blue) overlaps with the placodal domain of Sox2 (green). (*i–l*) *Xenopus* embryos at stage 25 (anterior to the right, posterior to the left). Diagram illustrating the plane of section (green line; *i*). Single *in situ* hybridization for Foxi4.1 (*j*), Sox10 (*k*) and MMP28 (*l*). Arrows show neural crest streams; arrowheads show epibranchial placodes; b, brain. Scale bars, 0.25 mm. Number of embryos per experiment greater than 20.

is upregulated at the end of gastrulation (St. 12.5) at which point it becomes detectable by *in situ* hybridization. We observed that MMP28 is a secreted MMP uniquely expressed at the neurula stage in the pre-placodal region adjacent to the prospective NC territory.

We showed that MMP28 expression is limited to the neural plate border at early neurula stage, then to the lateral placodes. This placodal expression is sustained in the epibranchial placodes at tail bud stage. In addition, MMP28 expression is detected along the migratory pathway of the first NC stream. As indicated earlier, MMP28 was first isolated in a screen for genes activated in the NC tissue at neurula stage [12]. However, as discussed by the authors of this study, because the microarray-based approach was performed on manually dissected embryonic tissues at different stages, potential contamination by surrounding tissues could not be excluded. In fact, the authors reported that MMP28 transcripts were detected at the edge of the NC domain, not the NC *per se* [12]. At neurula stage, our double *in situ* hybridization show that MMP28-positive signal does not overlap with the NC marker Snai2/Slug, but co-localizes with the lateral placodal domain of Sox2. While we have detected expression in the medial NC, overall MMP28 expression is primarily confined to the cranial placodes at early neurula stages.

The fact that MMP28 is strongly expressed in placodes is very interesting and unique. MMP1, 2, 3, 7, 9, 11, 13, 14, 15, 18, 20 and 24 are expressed during *Xenopus* embryogenesis (table 1). Almost half of them are expressed by the NC or surrounding tissues and their derivatives, but none of them has been described in placode cells at the stages studied here. The expression profile of MMP28 over time is comparable to that of Six1. However, the MMP28-positive territory only represents a subdomain of Six1 [32]. Notably, MMP28 was not detected in the most anterior placodal domain giving rise to olfactory placodes. By contrast, MMP28 expression is maintained in the epibranchial placodes that originate from the lateral placodes (Sox2-positive territory) and later is detected in between migratory NC streams.

NC cells and cranial placodes cooperate to form the cephalic peripheral nervous system. NC cells give rise to glial cells while neurons have a dual origin, coming from either NC, placodes or both, depending on the cranial ganglion of interest [35]. Interestingly, NC and placode interaction involved in peripheral nervous system patterning starts during NC migration. In *Xenopus*, placodal cells are the source of stromal cell-derived factor 1 (Sdf1/Cxcl12), a key regulator of NC cell migration [36]. Sdf1 promotes NC cell motility, which in the context of the constraints of the developing head leads to migration of NC cells

towards the placodes [37]. However, physical interactions between the two cell types leads to a repulsion. This short-range repulsion mid-range attraction system, coined 'chase-and-run', leads to a sustained directional migration of both NC and placode cells towards the ventral regions of the face [38]. Placodal cells are less motile than NC cells and are progressively pushed aside while NC cells migrate ventrally. This leads to a pattern of accumulation of placode cells in between NC streams [38,39]. Therefore, interfering with NC migration leads to defects in placode patterning and this relationship is conserved in vertebrates [38,40–42]. The expression of MMP28, a secreted MMP, in the placodes prior to the onset for NC migration raises the possibility that this enzyme might be required in proper NC–placode interactions.

5. Conclusion

Overall our data indicate that the onset of MMP28 expression in placodes occurs after the NC and pre-placodal domains have been specified at the neural plate border. MMP28 expression is excluded from the anterior-most placodes and persists in epibranchial placodes, strongly suggesting a role for this molecule in NC–placode interactions at early stages of the cranial peripheral nervous system formation.

Data accessibility. Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.5x69p8d0t [43].

Authors' contributions. N.G. performed most of the laboratory work with help from J.-P.S.-J. N.G., E.T. and J.-P.S.-J. designed the study, participated in data analysis, prepared the figures and drafted the manuscript. All authors gave final approval for publication and agree to be held accountable for the work performed herein.

Competing interests. We declare we have no competing interests.

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